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1 **Expression of the UVR8 photoreceptor in different tissues reveals tissue-**
2 **autonomous features of UV-B signalling**

3

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20 **Running Title:**

21 UVR8 signalling in different tissues

22 **SUMMARY STATEMENT**

23 This work analyses how the UV-B specific photoreceptor UVR8 regulates signalling,
24 development and growth when expressed only in specific tissues. We show that early
25 steps of UVR8-dependent signalling, such as accumulation of the key regulatory
26 transcription factor HY5, occur strictly in tissue-autonomous fashion. In contrast,
27 complex UV-B-induced changes, including proper acclimation of adult plants requires
28 simultaneous signalling in the epidermal and mesophyll cells and/or inter-tissue
29 signalling.

30

31

32 **ABSTRACT (162 words)**

33

34 The Arabidopsis UV-B photoreceptor UV RESISTANCE LOCUS 8 (UVR8)
35 orchestrates the expression of hundreds of genes, many of which can be associated
36 with UV-B tolerance. UV-B does not efficiently penetrate into tissues, yet UV-B
37 regulates complex growth and developmental responses. To unravel to what extent
38 and how UVR8 located in different tissues contributes to UV-B-induced responses,
39 we expressed UVR8 fused to the YELLOW FLUORESCENT PROTEIN (YFP)
40 under the control of tissue-specific promoters in a *uvr8* null mutant background. We
41 show that (i) UVR8 localized in the epidermis plays a major role in regulating
42 cotyledon expansion, and (ii) expression of UVR8 in the mesophyll is important to
43 protect adult plants from the damaging effects of UV-B. We found that UV-B induces
44 transcription of selected genes, including the key transcriptional regulator
45 *ELONGATED HYPOCOTYL 5 (HY5)*, only in tissues that express UVR8. Thus we

46 suggest that tissue-autonomous and simultaneous UVR8 signalling in different tissues
47 mediates, at least partly, developmental and defence responses to UV-B.

48

49

50 **Key words:**

51 Arabidopsis, ultraviolet-B, UVR8, signalling, tissue specificity

52 INTRODUCTION

53 Plants must adapt to the environment to optimize growth and development for
54 survival and successful reproduction. Light is an essential environmental factor and
55 necessary not only for photosynthesis but also as a signal for proper development and
56 growth. Plants evolved various photoreceptors that are able to monitor changes in the
57 quantity and quality of the ambient light environment. These include the blue/UV-A
58 light absorbing phototropins, cryptochromes and Zeitlupe family receptors; the
59 red/far-red absorbing phytochromes (phyA-phyE), as well as the UV-B photoreceptor
60 UV RESISTANCE LOCUS 8 (UVR8) (Galvao & Fankhauser, 2015).

61 UV-B radiation (280-315 nm) is an integral part of sunlight reaching the
62 Earth's surface, as it is only partially absorbed by the stratospheric ozone layer. UV-B
63 can damage several macromolecules (DNA, proteins etc.) (Hollosoy, 2002). However,
64 UV-B also activates UVR8-dependent signal transduction pathways and triggers
65 responses that manifest as inhibition of hypocotyl elongation, reduction of leaf size,
66 entrainment of the circadian clock, modification of shade avoidance response,
67 alteration of phototropism, increased accumulation of photo-protective flavonoids and
68 increased survival under UV-B stress (Kliebenstein *et al.*, 2002; Brown *et al.*, 2005;
69 Favory *et al.*, 2009; Feher *et al.*, 2011; Morales *et al.*, 2013; Hayes *et al.*, 2014;
70 Jenkins, 2014; Vandenbussche *et al.*, 2014).

71 At the cellular level the UVR8 photoreceptor can be detected both in the
72 cytoplasm and the nucleus in visible light, but irradiation with UV-B increases
73 accumulation of UVR8 in the nucleus (Kaiserli & Jenkins, 2007; Yin *et al.*, 2016).
74 Nuclear localization of UVR8 is required but not sufficient for UV-B signalling
75 (Brown *et al.*, 2005; Kaiserli & Jenkins, 2007; Yin *et al.*, 2016). It is a matter of
76 debate whether or not UVR8 directly associates with chromatin to regulate UV-B-

77 dependent transcription of target genes, including *HY5* (Cloix & Jenkins, 2008;
78 Binkert *et al.*, 2016). The ELONGATED HYPOCOTYL 5 (HY5) transcription factor
79 is a major positive regulator of photomorphogenesis both in visible (Lee *et al.*, 2007)
80 and UV-B light (Ulm *et al.*, 2004; Brown *et al.*, 2005; Oravecz *et al.*, 2006; Binkert *et*
81 *al.*, 2014). *hy5* mutants are largely impaired in UV-B-responsive gene expression and
82 the accumulation of UV-B-protective flavonoid pigments, leading to reduced UV-B
83 tolerance and survival (Brown *et al.*, 2005; Oravecz *et al.*, 2006; Stracke *et al.*, 2010).
84 UV-B irradiation was shown to rapidly induce *HY5* gene expression (Ulm *et al.*, 2004;
85 Brown & Jenkins, 2008; Binkert *et al.*, 2014; Binkert *et al.*, 2016) and the
86 accumulation of HY5 protein in the nucleus (Oravecz *et al.*, 2006).

87 UV-B penetrates rather poorly into tissues below the epidermis. Indeed, leaf
88 epidermal transmittance of UV-B is less than 10 %, measured in many different
89 species under various circumstances (Robberecht *et al.*, 1980; Day *et al.*, 1993;
90 Markstadter *et al.*, 2001; Qi *et al.*, 2003; Nybakken *et al.*, 2004). UVR8 is expressed
91 ubiquitously in different organs of mature *Arabidopsis* (Rizzini *et al.*, 2011), but the
92 precise distribution pattern and the accumulation level of the photoreceptor in various
93 tissues have not yet been investigated. It follows that it is not understood how the
94 action of UVR8 in different tissues/organs is integrated to regulate complex
95 physiological responses as hypocotyl growth inhibition or leaf size, and how the
96 strongly varying UV-B intensities in different tissues modulate UVR8-dependent
97 signalling.

98 Here we characterized the spatio-temporal aspects of UV-B-induced, UVR8-
99 mediated signalling to provide insight into the molecular mechanism mediating signal
100 integration between different tissues/organs. We first determined the distribution
101 pattern and level of YFP-UVR8 under the control of its own promoter. Next we

102 characterized to what extent UV-B-induced physiological and molecular responses are
103 mediated by tissue-autonomous and/or inter-tissue signalling in transgenic lines that
104 expressed the photoreceptor in a tissue-specific fashion. Our data suggest that UVR8
105 responses are mediated partly by tissue-autonomous signalling, but proper regulation
106 of hypocotyl growth inhibition and establishment of UV-B tolerance require either
107 UVR8 action in different tissues and/or inter-tissue signalling.

108

109 **MATERIALS AND METHODS**

110 **Molecular cloning**

111 The coding region of *YFP* and *UVR8* were cloned into the pPCV812 plasmid
112 (Bauer *et al.*, 2004) as *SmaI-EcoRI* and *EcoRI-SacI* fragments, respectively. The
113 *MERISTEM LAYER 1* (*ProML1*), *SUCROSE /H⁺ SYMPORTER 2* (*ProSUC2*) and
114 *CHLOROPHYLL A/B BINDING PROTEIN 3* (*ProCAB3*) promoter fragments were
115 cloned as described by Kirchenbauer *et al.* (2016) whereas the *ProUVR8* was inserted
116 as a 2569 bp *Sall-BamHI* fragment including the 5' leader sequence. The coding
117 sequence of the β -glucuronidase (*GUS*) as a *SmaI-XhoI* fragment (Adam *et al.*, 1995),
118 *GFP* as a *XhoI-ClaI* fragment and *NLS* as a *ClaI-SacI* fragment (Wolf *et al.*, 2011)
119 were cloned into the *pPCVB812* binary vector (Bauer *et al.*, 2004) resulting in *GUS*-
120 *GFP-NLS pPCVB*. This vector was digested with *HindIII* and *SmaI* restriction
121 enzymes and the *ProHY5* (Oravec *et al.*, 2006) was inserted as a *HindIII-StuI*
122 fragment replacing the *Pro35S* promoter. *ProELIP2* and *ProPRR9* were cloned as
123 2772 bp (*BamHI-XbaI*) and 1324 bp (*BamHI-SmaI*) fragments including the 5' leader
124 sequences, respectively. Cloning of *ProHY5:HY5-GFP* was described in detail by
125 Kirchenbauer *et al.* (2016).

126

127 **Plant material**

128 Throughout the study we used the *Arabidopsis thaliana* L (Heynh.) *uvr8-6* null
129 mutant (Favory *et al.*, 2009), with the Columbia accession as wild type (WT) control.
130 We raised 10 independent transgenic lines per construct and selected those which
131 segregated the transgene as a single Mendelian trait. At least 3 independent lines were
132 studied and comparable results are presented. Arabidopsis transformation, principles
133 of selection and handling of transgenic lines were described earlier in detail
134 (Kirchenbauer *et al.*, 2016).

135

136 **Seedling growth conditions and light treatments**

137 Seeds were surface sterilized and subsequently stratified for 72 h in the dark (4
138 °C) on ½ Murashige and Skoog (MS) medium (Sigma-Aldrich, Budapest, Hungary)
139 containing 1% sucrose and 0.8% agar. For microscopic analysis, the seedlings were
140 grown in 12 h white light (WL, 80 $\mu\text{mol m}^{-2} \text{s}^{-1}$)/12 h dark at 22 °C for 6 days (MLR-
141 350, Sanyo, Gallenkamp, UK) and then placed under continuous white light
142 supplemented with UV-B for 16 h at 22 °C. White light was produced by PHILIPS
143 TL-D 18W/33-640 tubes (10 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Non-damaging photomorphogenic (low-
144 fluence) UV-B was produced by PHILIPS ULTRAVIOLET-B TL20W/01RS tubes
145 (1.5 $\mu\text{mol m}^{-2} \text{s}^{-1}$). To modulate UV-B light we used 3-mm thick transmission cut-off
146 filters of the WG series (Schott, Mainz, Germany), as described previously (Ulm *et*
147 *al.*, 2004). UV-B treated seedlings (+UV-B) were covered with WG305 filter with
148 half-maximal transmission at 305 nm, whereas non-UV-B irradiated control seedlings
149 were covered with WG385 filter with half-maximal transmission at 385 nm (-UV-B)
150 as applied in work published earlier (Oravecz *et al.*, 2006; Favory *et al.*, 2009; Rizzini
151 *et al.*, 2011). UV-B was measured with a VLX-3W UV light meter equipped with a

152 CX-312 sensor (Vilber Lourmat, Eberhardzell, Germany) and the visible part was
153 measured with an LI-250 Light Meter (Li-Cor, Lincoln, NE, USA). For hypocotyl and
154 cotyledon measurements, seedlings were grown for 3 days in light/dark chambers
155 before being exposed to continuous WL supplemented with UV-B for 4 days or 5
156 days.

157

158 **Microscopy techniques**

159 Confocal laser scanning microscopy (CLSM) settings and quantification of
160 nuclear fluorescence were described in detail by Kirchenbauer *et al.* (2016)

161

162 **Flavonoid detection using confocal laser scanning microscopy**

163 Seeds were stratified and germinated as described above. Seedlings were
164 grown for 2 days in 12 h light/12 h dark chambers and were placed under 1.5 μmol
165 $\text{m}^{-2} \text{s}^{-1}$ WL supplemented with 1.5 $\mu\text{mol m}^{-2} \text{s}^{-1}$ UV-B light for 4 days. Seedlings
166 treated with UV-B were covered with a WG305 filter, whereas the negative controls
167 (-UV-B) were covered with WG385. Prior to microscopic analysis seedlings were
168 incubated in 0.1% (w/v) Naturstoffreagenz A (DPBA, Sigma-Aldrich) in 0.15 M
169 phosphate buffer (pH 6.8) in the dark. After 15 min incubation time DPBA was
170 removed by exchanging the buffer for fresh phosphate buffer twice. CLSM was used
171 to detect DPBA-flavonoid specific fluorescence (488 nm laser; pinhole: 200 μm ;
172 spectral emission detector: 501-601 nm).

173

174 **Hypocotyl length and cotyledon area measurements**

175 Measurements of hypocotyl length and cotyledon area were performed as
176 described earlier (Adam *et al.*, 2013). At least 40 seedlings (hypocotyl length) or 100

177 cotyledons were measured for each line and each treatment. Ratios of UV-B
178 treated/non-treated hypocotyl lengths and cotyledon areas were calculated in each
179 experiment. Experiments were repeated at least three times. The calculated ratio
180 values were averaged and the standard error values of the means were obtained and
181 plotted.

182

183 **Protein isolation and western blot**

184 Preparation of plant protein extracts and western blotting were described by
185 Bauer *et al.* (2004). Application of anti-UVR8, anti-ACTIN antibodies and signal
186 processing were also described earlier (Heijde & Ulm, 2013; Medzihradsky *et al.*,
187 2013). All protein extraction and western blotting was repeated three times and a
188 representative image is presented. Signal quantification was made using Image J
189 software (NIH).

190

191 **Determination of transcript levels**

192 Total RNA isolation, cDNA synthesis and quantitative RT-PCR analysis were
193 performed as described by Feher *et al.* (2011).

194

195 **Propagation and UV-B treatment of adult plants for phenotype analysis and** 196 **chlorophyll determination**

197 Arabidopsis seeds were sown on soil, stratified for three days at 4 °C and then grown
198 in a climate-controlled growth chamber (Grobank, CLF Plant Climatics, Werten,
199 Germany) in short days conditions (8 h light / 16 h dark) under WL ($120 \mu\text{mol m}^{-2}$
200 s^{-1}) or WL supplemented with UV-B at 22°C. The visible part was measured with an
201 LI-250 Light Meter (Li-Cor). The light conditions in the chambers were set following

the general guidelines described by Aphalo *et al.* (2012) and the full spectra of the applied light was analysed with a QE65000 spectrometer (Ocean Optics, Dunedin, FL, USA) (Figure S1). We used white fluorescent tubes (Osram L18W) and the same type narrowband UV-B tubes, what were used in the seedling irradiation treatments (TL20W/01RS, Philips) without plastic filtering. The applied UV-B fluence rates (2 or 12 $\mu\text{mol m}^{-2} \text{s}^{-1}$) were comparable to the natural values measured in Szeged, Hungary on an average sunny summer day (7-15 $\mu\text{mol m}^{-2} \text{s}^{-1}$ between 11:00-13:00 CET on 09.06.2010.). UV-B was measured with a VLX-3W UV light meter equipped with a CX-312 sensor (Vilber Lourmat). Rosette diameter was quantified in images of 7-week-old plants using ImageJ. Three repetitions of each experiment were performed using two independent lines for the tissue-specific lines. At least four plants were measured in each repetition for each genotype and independent line. Determination of chlorophyll levels were described earlier (Porra *et al.*, 1989)

215

216

217 **RESULTS**

218 **Expression of the *ProUVR8:YFP-UVR8* transgene is restricted to epidermal and** 219 **mesophyll cells**

220 To address where UVR8 is expressed, we generated transgenic lines expressing the
221 *YFP-UVR8* fusion protein under the control of its own promoter in a *uvr8* null mutant
222 background and determined its expression pattern by using CLSM. We found that the
223 *UVR8* promoter drives the expression of YFP-UVR8 in the epidermal and, to a lesser
224 extent, the mesophyll/subepidermal cells of cotyledons and hypocotyls (Figures 1A-C,
225 S2-S4). Accumulation of the YFP-UVR8 fusion protein was below detection level in
226 the vascular bundles. But it should be noted that the YFP-UVR8 amount corresponded

227 to ~10% of the native UVR8 protein detected in WT seedlings (Figure 2A) and that
228 we did not identify any *ProUVR8:YFP-UVR8* line with higher YFP-UVR8 protein
229 amounts.

230

231 **Characterization of transgenic lines expressing YFP-UVR8 in selected tissues**

232 To assess the function of UVR8 located in different tissues we expressed YFP-UVR8
233 in the *uvr8* mutant background under the control of *ProML1*, *ProSUC2* and *ProCAB3*
234 promoters that have already been used in numerous studies to express proteins of
235 interest in epidermal, companion and mesophyll cells, respectively (Mitra *et al.*, 1989;
236 Sessions *et al.*, 1999; Srivastava *et al.*, 2008; Kirchenbauer *et al.*, 2016). Figure 1D-I
237 and Figures S2-S4 demonstrate that the *ProML1* drives the expression of YFP-UVR8
238 selectively in epidermal cells, whereas *ProCAB3* in the sub-epidermal (mesophyll)
239 cells of cotyledons and hypocotyls. As expected, no activity of these promoters was
240 detected in the vascular bundles. By contrast, *ProSUC2* expressed YFP-UVR8 in the
241 vasculature and sub-epidermal cells of cotyledons and hypocotyls (Figure 1J-L and
242 S2-S4). Western blot analysis showed that the total amount of YFP-UVR8 in
243 *ProML1:YFP-UVR8* was ~5%, in *ProSUC2:YFP-UVR8* ~25% and in *ProCAB3:YFP-*
244 *UVR8* 75% of the amount of endogenous UVR8 in WT seedlings (Figure 2A). To
245 facilitate direct comparison of the level of YFP-UVR8 in different cell types, we
246 monitored its accumulation by CLSM. The amount of YFP-UVR8 was (i) comparable
247 in the epidermal cells of *ProUVR8:YFP-UVR8* and *ProML1:YFP-UVR8*, (ii) about 4-
248 5-fold lower in the mesophyll cells of *ProUVR8:YFP-UVR8* as compared to
249 *ProCAB3:YFP-UVR8* and about the same in *ProSUC2:YFP-UVR8* (Figure S5). It was
250 not feasible to compare its accumulation by this method in the vascular bundles.

251

Complementation of seedling phenotypes of the *uvr8-6* mutant by tissue-specific expression of YFP-UVR8

To assess the function of UVR8 in different tissues, we measured typical photomorphogenic responses such as inhibition of hypocotyl elongation and expansion of cotyledons, of the various transgenic seedlings exposed to UV-B irradiation. Figure 2B shows that supplemental narrowband UV-B inhibited hypocotyl growth in the wild-type seedlings, whereas the *uvr8* mutant seedlings were much less responsive, in agreement with previous results (Favory *et al.*, 2009). All transgenic seedlings, except *ProSUC2:YFP-UVR8*, showed pronounced UV-B-induced hypocotyl growth inhibition, but did not fully complement the phenotype of the *uvr8* mutant (Figure 2B). We also measured the changes of cotyledon area caused by UV-B irradiation. Figure 2C illustrates that UV-B irradiation decreased the cotyledon size of the *uvr8*, *ProCAB3:YFP-UVR8* and the *ProSUC2:YFP-UVR8* seedlings, whereas the same UV-B treatment slightly increased the cotyledon size in the *ProUVR8:YFP-UVR8*, *ProMLI:YFP-UVR8* and wild-type plants.

The above results indicate that (i) the YFP-UVR8 fusion protein is a functional photoreceptor, confirming previous reports (Brown *et al.*, 2005; Kaiserli & Jenkins, 2007; Huang *et al.*, 2014; Binkert *et al.*, 2016); (ii) UVR8 signalling contributes to UV-B-induced inhibition of hypocotyl growth both in the epidermal and mesophyll cells; (iii) UVR8 expression in the epidermis is necessary for proper cotyledon expansion under UV-B light; and (iv) YFP-UVR8 expressed in vascular bundles plays a very limited role, if any, in regulating hypocotyl growth and cotyledon expansion (Table S1).

The UV-B-induced, UVR8-regulated induction of *HY5* is tissue-autonomous

277 Increase in the mRNA level and nuclear accumulation of the key UV-B signal
 278 transduction component HY5 are among the early steps of the UV-B-induced
 279 signalling cascade initiated by UVR8 (Ulm *et al.*, 2004; Brown *et al.*, 2005; Oravecz
 280 *et al.*, 2006). To examine the tissue specificity of these responses, we introduced the
 281 *ProHY5:HY5-GFP* (to determine the cell-specific accumulation of HY5 protein) and
 282 *ProHY5:GUS-GFP-NLS* (to determine the cell-specific induction of *HY5*
 283 transcription) reporters into transgenic *uvr8* mutant lines expressing YFP-UVR8 in
 284 different tissues. Figure 3 demonstrates that (i) the abundance of HY5-GFP was low
 285 in seedlings grown in white light, and (ii) UV-B irradiation promoted accumulation of
 286 HY5-GFP only in those cells which also contained detectable amounts of YFP-UVR8.
 287 Similarly, we found that the UVR8-dependent induction of *HY5* transcription is also
 288 restricted to YFP-UVR8-containing cells (Figure S6). Thus our results indicate that
 289 regulation of the expression of *HY5* by UVR8 is a tissue-autonomous response.

290

291 **UV-B induction of the transcription of *HY5*-dependent and -independent genes is**
 292 **controlled by UVR8 in a tissue-autonomous fashion**

293 To get more insight into the tissue-related organization of UVR8 signalling,
 294 we also introduced the *ProELIP2:GUS-GFP-NLS* and *ProPRR9:GUS-GFP-NLS*
 295 transgenes into the *ProUVR8:YFP-UVR8*, *ProMLI:YFP-UVR8* and *ProCAB3:YFP-*
 296 *UVR8* expressing lines. The *EARLY LIGHT-INDUCED PROTEIN 2 (ELIP2)* is
 297 involved in the photoprotection of thylakoid membranes (Hutin *et al.*, 2003). UV-B
 298 irradiation induces accumulation of *ELIP2* mRNA (Ulm *et al.*, 2004; Feher *et al.*,
 299 2011), and this response requires functional UVR8 and HY5 (Figure S7) (Oravecz *et*
 300 *al.*, 2006; Favory *et al.*, 2009). Figure 4 demonstrates that the activity of *ProELIP2* is
 301 low in white light, and that UV-B irradiation strongly enhances its activity only in

302 those cells which also contain detectable amounts of YFP-UVR8, indicating that the
303 photoreceptor regulates HY5-dependent expression of *ELIP2* in a tissue-autonomous
304 fashion.

305 *PSEUDO-RESPONSE REGULATOR 9 (PRR9)* is a component of the plant
306 circadian clock (Nakamichi *et al.*, 2005). UV-B-induction of *ProPRR9* depends on
307 UVR8 (Feher *et al.*, 2011), but it is independent of HY5 (Figure S7). In contrast to the
308 *HY5* and *ELIP2* promoters, *ProPRR9* was active in the sub-epidermal cells of
309 cotyledons in transgenic plants grown in white light. UV-B strongly induced
310 *ProPRR9* activity only in those sub-epidermal cells that contained detectable amounts
311 of YFP-UVR8 (Figure S8). Elevated expression of *ProPRR9:GUS-GFP-NLS* was not
312 detectable in the epidermis of *ProMLI:YFP-UVR8* and *ProUVR8:YFP:UVR8* lines,
313 although these cells express YFP-UVR8.

314

315 **UVR8-dependent flavonoid accumulation occurs in a tissue-autonomous fashion**

316 DPBA forms complexes with flavonoid compounds, which can be visualized by
317 CLSM (Schnitzler *et al.*, 1996; Hutzler *et al.*, 1998; Peer *et al.*, 2001). We applied an
318 irradiation protocol which allowed detectable accumulation of flavonoids under
319 supplemental UV-B in wild-type but not in *uvr8* seedlings. We detected the highest
320 level of UV-B-induced flavonoid accumulation on the inner side of the adaxial
321 epidermal cells in WT seedlings (Figure 5), as previously reported (Hutzler *et al.*,
322 1998; Agati *et al.*, 2011). Moreover, all YFP-UVR8-expressing lines accumulated
323 flavonoids UV-B-dependently, with a similar accumulation pattern but to lower levels
324 than WT.

325

326 **Adult plants require UVR8 in the mesophyll cells for proper acclimation and**
327 **survival under UV-B**

328 UVR8 plays a role not only at the seedling stage but also in acclimation to
329 UV-B of adult plants (Favory *et al.*, 2009). We examined 7-week-old plants
330 expressing YFP-UVR8 in different tissues and found that all plants developed equally
331 without UV-B (Figure 6A). Weak supplemental UV-B triggered rosette growth
332 inhibition and shortening of petioles in the wild-type plants, whereas the *uvr8-6*
333 mutant showed a very limited rosette growth reduction and developed light green
334 leaves, indicating that this dose of UV-B elicited mainly UVR8 photoreceptor-
335 mediated photomorphogenic responses (Figure 6A). Thus, *uvr8* mutants are
336 hyposensitive to UV-B considering UV-B-induced photomorphogenesis and
337 acclimation, as previously reported (Favory *et al.*, 2009). Transgenic lines expressing
338 YFP-UVR8 displayed WT-like acclimation (Figure 6A), with comparable rosette
339 development (Figure 6B) and chlorophyll accumulation (Figure 6C), except for the
340 *ProCAB3:YFP-UVR8* plants, which had small rosettes (Figure 6B) and accumulated
341 chlorophyll to higher levels (Figure 6C).

342 Stronger supplemental UV-B was lethal to *uvr8* mutants that, in contrast to wild
343 type, were not able to acclimate to UV-B (Figure 6A). Thus under these conditions
344 the effect of UV-B acclimation on UV-B tolerance can be assayed. Next to wild type,
345 also the *ProUVR8:YFP-UVR8* and *ProMLI:YFP-UVR8* lines survived the higher UV-
346 B levels but developed smaller rosettes. The *ProCAB3:YFP-UVR8* and
347 *ProSUC2:YFP-UVR8* plants displayed a strong over-expression phenotype
348 characteristic for plants producing high amounts of UVR8 under the control of
349 constitutive promoters (Favory *et al.*, 2009; Heijde *et al.*, 2013; Fasano *et al.*, 2014).
350 We found that these lines indeed over-expressed UVR8 in adult plants grown on soil

351 as compared with WT (Figure S9). Taken together, these results indicate that the
352 expression of UVR8 in subepidermal or epidermal tissues efficiently facilitates
353 acclimation and survival under UV-B.

354

355 **DISCUSSION**

356 Analysis of transgenic *ProUVR8:YFP-UVR8* plants revealed the presence of
357 the YFP-UVR8 fusion protein in the epidermal and sub-epidermal cells of cotyledons
358 and hypocotyls of seedlings exposed to UV-B. However, in these lines YFP-UVR8
359 accumulated to levels lower than endogenous UVR8, thus we cannot exclude the
360 presence of low amounts of UVR8 in the vascular tissues of WT seedlings. The fusion
361 protein was biologically active, since the *ProUVR8:YFP-UVR8* transgenic seedlings
362 and adult plants displayed partially or fully complemented UV-B responses. Thus we
363 assume that UVR8 signalling does not play a role in the vasculature, independently of
364 the developmental stage.

365 *ProMLI:YFP-UVR8* displayed fully complemented UV-B-induced cotyledon
366 expansion and partially restored hypocotyl growth inhibition, suggesting that
367 epidermal UVR8 is critical for the regulation of these responses. *ProCAB3:YFP-*
368 *UVR8* seedlings containing high levels of YFP-UVR8 in the subepidermal cells also
369 displayed a partially complemented hypocotyl growth inhibition but a non-
370 complemented cotyledon phenotype. The *ProSUC2:YFP-UVR8* line, despite the fact
371 that it contained a relatively high amount of fusion protein, failed to complement
372 cotyledon growth and displayed only a weak hypocotyl growth inhibition response
373 (Table S1, Figure 2). The latter could be the result of the UVR8 action in mesophyll
374 cells rather than in the vasculature. Based on these data we conclude that at the
375 seedling stage the primary sites of UV-B perception are the epidermis and, to a lesser

376 extent, the mesophyll/sub-epidermal cells. The low penetration of UV-B into deeper
377 layers of plant organs (Day *et al.*, 1993), lends further support to the above
378 conclusion. The distinguished role of epidermis in regulating hypocotyl growth is not
379 unique to UVR8 action, as similar data were reported for phyA (Kirchenbauer *et al.*,
380 2016) phyB (Endo *et al.*, 2005; Kim *et al.*, 2016) and brassinosteroid signalling
381 (Savaldi-Goldstein *et al.*, 2007). However, both Kirchenbauer *et al.* (2016). and
382 Savaldi-Goldstein *et al.* (2007) concluded that exclusive action of phyA or
383 brassinosteroid signalling in the epidermis is not sufficient to recapitulate full
384 regulation of this response. Therefore we assume that the UVR8-mediated inhibition
385 of hypocotyl growth is also mediated partly by the simultaneous action of UVR8 in
386 various tissues and/or inter-tissue signalling.

387 Adult *ProCAB3:YFP-UVR8* and *ProSUC2:YFP-UVR8* plants having high
388 levels of YFP-UVR8 in the mesophyll displayed an over-expression phenotype,
389 whereas the phenotype of *ProUVR8:YFP-UVR8* plants was similar to WT when
390 exposed to strong UV-B (Figure 6). Although subepidermal/mesophyll cells also
391 contain flavonoids (Agati *et al.*, 2011) we do not attribute the over-expression
392 phenotype directly to the accumulation of flavonoids in these cell types. However, we
393 assume that (i) UVR8 in the mesophyll is required for maintaining photosynthetic
394 efficiency under elevated UV-B (Davey *et al.*, 2012) maybe by regulating the levels
395 of the D1 and D2 core proteins, as described recently in *Chlamydomonas* (Tilbrook *et*
396 *al.*, 2016), and that (ii) this process needs UVR8 located in cells containing
397 chloroplasts. As for proper rosette development, the phenotypes of the *ProML1:YFP-*
398 *UVR8* and *ProUVR8:YFP-UVR8* lines suggest that together with the mesophyll
399 UVR8, the action of epidermal UVR8 is still required. As for the *ProSUC2:YFP-*
400 *UVR8* plant, it remains to be seen whether the activity of UVR8 in the vasculature

401 contributes to the acclimation response, or it is due to *ProSUC2* promoter action in
402 subepidermal cells. Taken together, we conclude that in mature plants, simultaneous
403 signalling in the epidermal and mesophyll cells and/or inter-tissue signalling is
404 required to optimise growth and development under UV-B.

405 UV-B-induced flavonoid accumulation both in the epidermis (*ProML1:YFP-*
406 *UVR8* or *ProUVR8:YFP-UVR8*) and mesophyll cells (*ProCAB3:YFP-UVR8* or
407 *ProSUC2:YFP-UVR8*) appears to be regulated by YFP-UVR8 located in the same
408 tissue, i.e. in a tissue-autonomous fashion (Figure 5). However, at present the
409 contribution of inter-tissue signalling or transport of flavonoids (Buer *et al.*, 2007) in
410 regulating their accumulation can not be ruled out.

411 To provide a mechanistic explanation for UV-B-induced developmental
412 responses, we examined the expression patterns of various genes shown to be
413 regulated by UVR8. UV-B-induced transcription and accumulation of the key
414 regulator HY5 was restricted to cells containing UVR8 (Figures 3, S6). The phyA
415 photoreceptor was also shown to regulate *HY5* expression in a similar fashion
416 (Kirchenbauer *et al.*, 2016). These data suggest that far-red and UV-B light regulated
417 expression of *HY5*, probably an early, rate-limiting step of both signal transduction
418 cascades, is mediated in a tissue-autonomous fashion by both photoreceptors.
419 Similarly to *HY5*, the UV-B-induced expression of *ELIP2* which requires functional
420 *HY5* and that of *PRR9* whose expression is not regulated by *HY5* occurs in a strictly
421 tissue-autonomous way (Figures 4, S8).

422 Taken together, we found no evidence at the molecular level that UVR8-
423 signalling initiates signal crosstalk between different tissues. However, it was reported
424 that UV-B irradiation of certain parts of the plants results in changes of gene
425 expression in shielded organs, indicating that UV-B-induced inter-organ signalling

can occur in higher plants (Casati & Walbot, 2004). Therefore we hypothesize that inter-tissue signalling, mediated by yet unknown mobile compounds contributes to the manifestation of UVR8-regulated responses. For example, it was reported that HY5 regulates auxin signalling under different light treatments including UV-B irradiation (Cluis *et al.*, 2004; Sibout *et al.*, 2006; Hayes *et al.*, 2014; Vandenbussche *et al.*, 2014). However, to unravel the molecular aspects of UVR8-modulated hormone signalling requires the development of new cellular markers.

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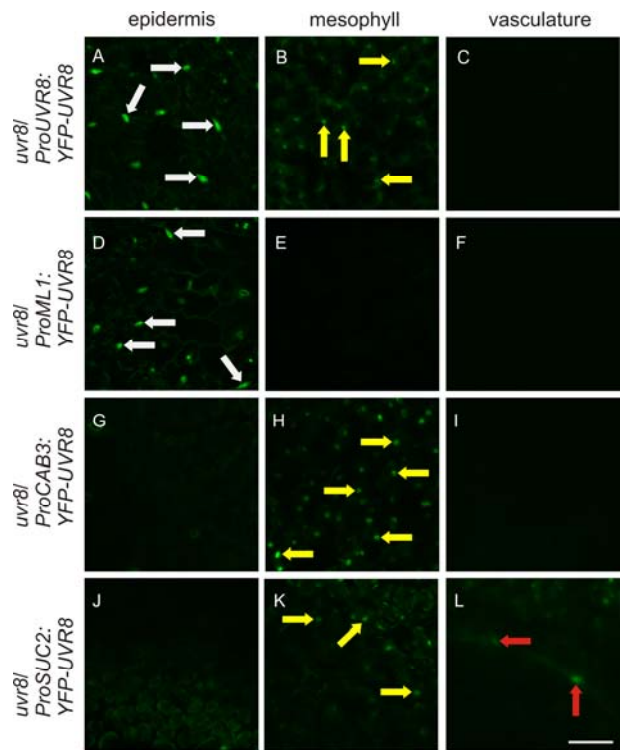
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634

635 **FIGURES**



636
637 **Figure 1**

638 **Tissue-specific expression of YFP-UVR8 in the cotyledons of transgenic *uvr8-6***
639 **seedlings.**

640 Localization of the YFP-UVR8 fusion protein was monitored by CLSM in the
641 cotyledons of seedlings grown in constant WL supplemented with UV-B. To facilitate
642 comparison of the expression levels of YFP-UVR8 in the examined transgenic lines,
643 images representing the same tissue were obtained using identical microscope
644 settings. The epidermis (A, D, G, J), the sub-epidermal mesophyll cells (B, E, H, K)
645 and the vasculature (C, F, I, L) of seedlings expressing *ProUVR8:YFP-UVR8*, (A, B,
646 C) or *ProML1:YFP-UVR8* (D, E, F) or *ProCAB3:YFP-UVR8* (G, H, I) or
647 *ProSUC2:YFP-UVR8* (J, K, L) were examined. White arrows mark positions of
648 selected nuclei in the epidermis, yellow arrows point to nuclei in the mesophyll,
649 whereas red arrows indicate nuclei/cells in the vasculature. Scale bar = 50 μ m.

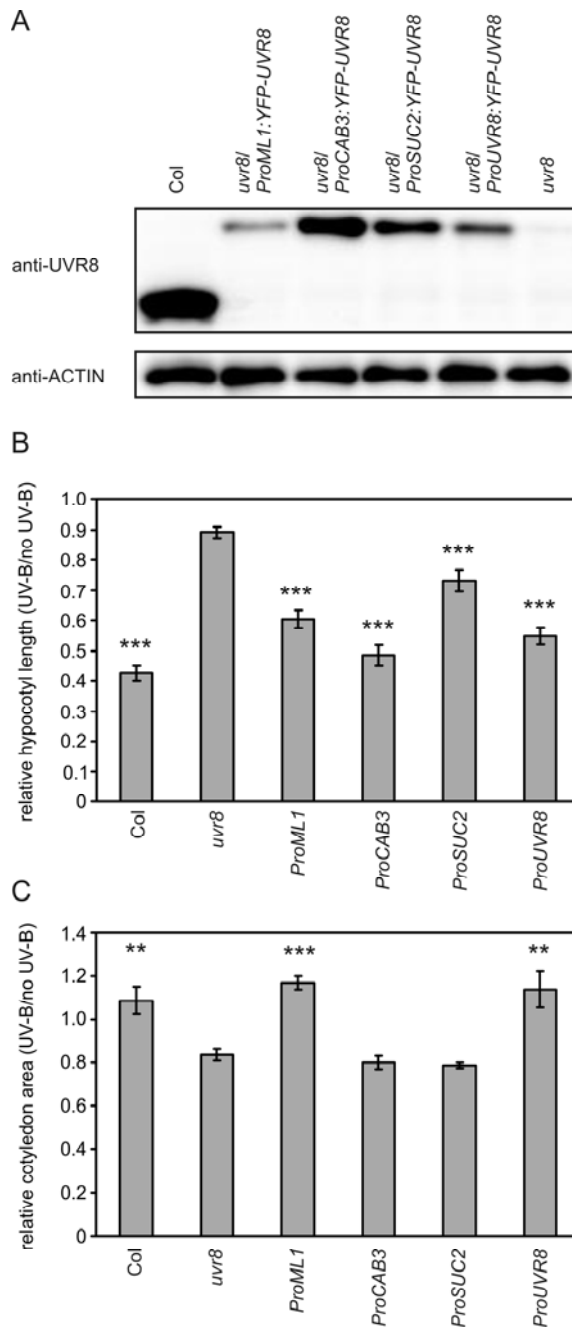


Figure 2

Expression and mutant phenotype complementation of YFP-UVR8 in *uvr8-6*

A. Determination of endogenous and YFP-UVR8 protein levels.

Total protein extract was isolated from 4-day-old seedlings grown under constant WL supplemented with UV-B. The proteins were detected using UVR8-specific antibody (anti-UVR8). The blot was reprobed with anti-ACTIN antibody as loading control.

657 **B. Effect of UV-B on hypocotyl length.**

658 Hypocotyl lengths of seedlings irradiated with constant WL supplemented with (UV-
659 B) or without (no-UV-B) UV-B for 4 days were measured and relative hypocotyl
660 lengths (UV-B/no-UV-B) were calculated. Each measurement was repeated 3 times;
661 error bars represent standard error of the mean. Lines: Col= Columbia wild type; *uvr8*
662 = *uvr8-6* mutant, *ProUVR8*= *ProUVR8:YFP-UVR8*; *ProML1*= *ProML1:YFP-UVR8*;
663 *ProCAB3*= *ProCAB3:YFP-UVR8*; *ProSUC2*= *ProSUC2:YFP-UVR8*. Each transgene
664 is expressed in the *uvr8-6* background. Asterisks mark lines that display significant
665 differences as compared with the *uvr8* mutant line calculated by the Student's t-test
666 (significance: *P< 0.05, ** P< 0.01, ***P < 0.005).

667 **C. Effect of UV-B on cotyledon expansion.**

668 Cotyledon areas of seedlings irradiated with constant WL supplemented with (UV-B)
669 or without (no UV-B) UV-B were measured and relative cotyledon areas (UV-B/no
670 UV-B) are plotted here. Each measurement was repeated 3 times; error bars represent
671 standard error of the mean. Lines: Col= Columbia wild type; *uvr8* = *uvr8-6* mutant,
672 *ProUVR8*= *ProUVR8:YFP-UVR8*; *ProML1*= *ProML1:YFP-UVR8*; *ProCAB3*=
673 *ProCAB3:YFP-UVR8*; *ProSUC2*= *ProSUC2:YFP-UVR8*. Each transgene is expressed
674 in the *uvr8-6* background. Asterisks mark lines that display significant differences as
675 compared with the *uvr8* mutant line calculated by the Student's t-test (significance:
676 *P< 0.05, ** P< 0.01, ***P < 0.005).

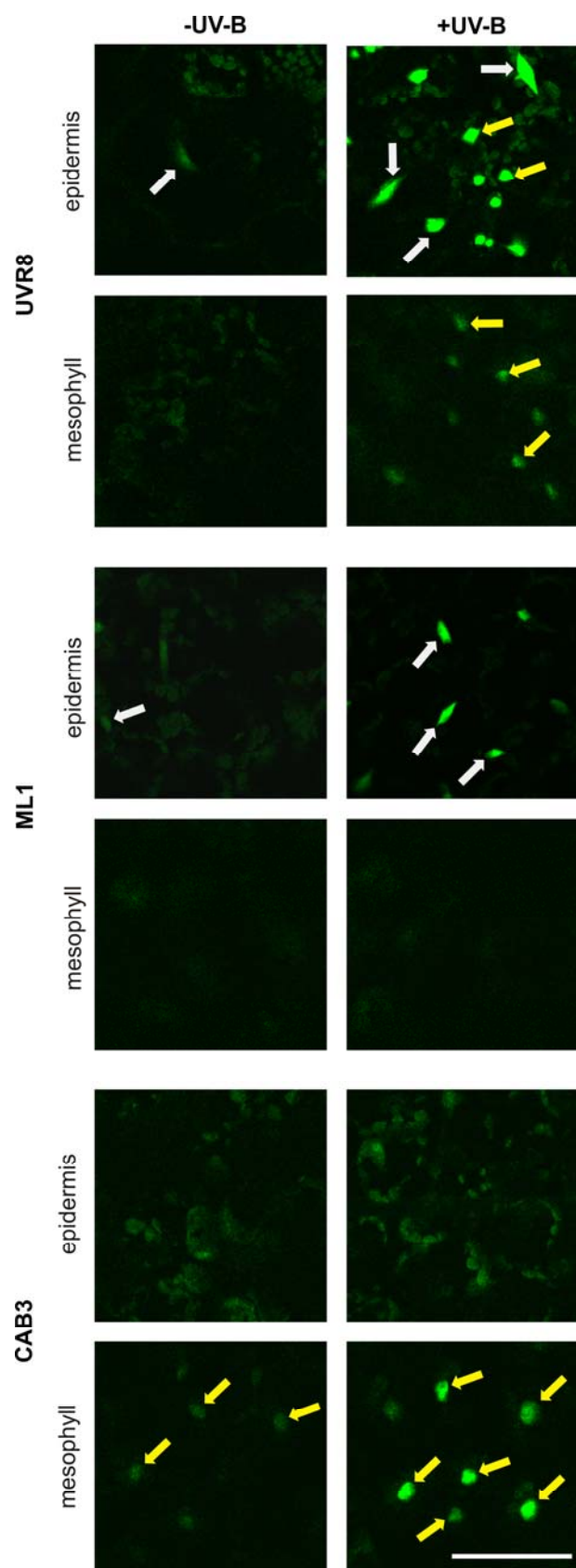
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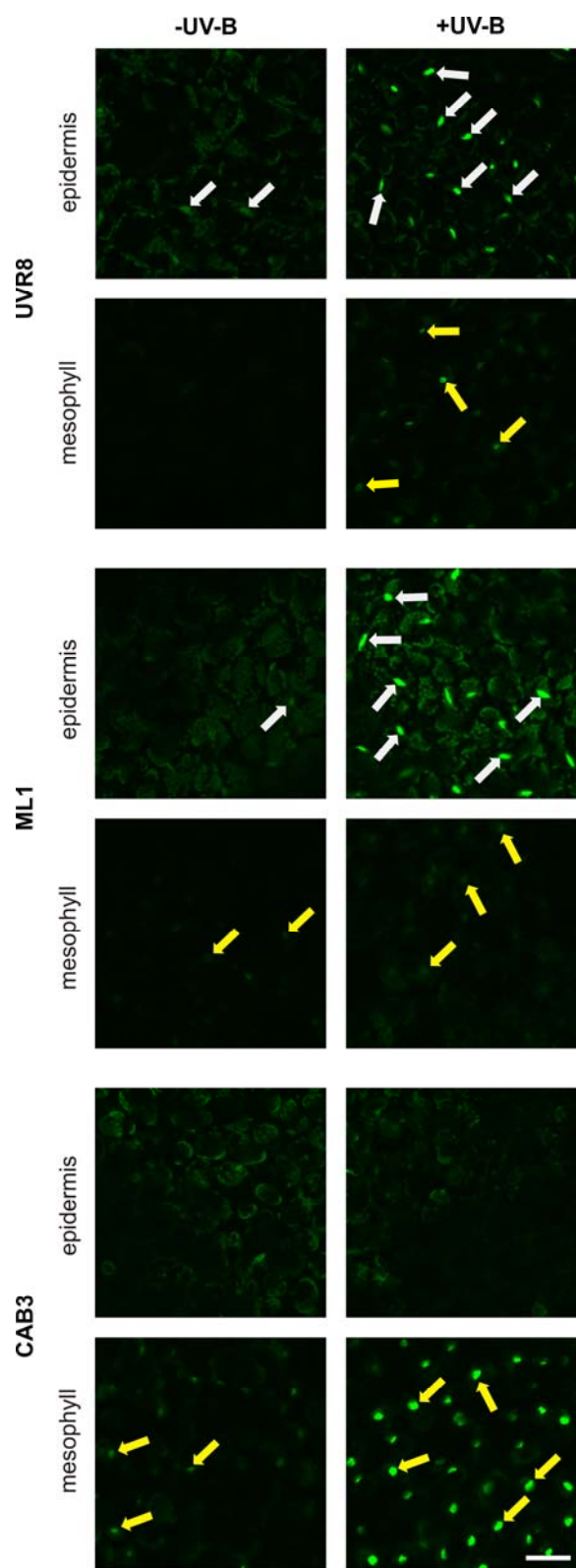
682

683 **Figure 3**

684 **UV-B induction of *ProHY5:HY5-GFP* in the cotyledon cells of transgenic lines**
685 **expressing YFP-UVR8 in different tissues.**

686 *ProHY5:HY5-GFP* was introduced into transgenic *uvr8* lines expressing
687 *ProUVR8:YFP-UVR8* (UVR8), *ProML1:YFP-UVR8* (ML1) or *ProCAB3:YFP-UVR8*
688 (CAB3). Localization of the HY5-GFP fusion protein was monitored by CLSM in the
689 epidermis and mesophyll cells of the cotyledon of 7-day-old seedlings irradiated with
690 constant WL supplemented with UV-B (+UV-B) or not supplemented (-UV-B).
691 Identical microscope settings were used to allow determination of the difference
692 between the visual signals of the +UV-B and -UV-B image pairs. White arrows mark
693 the positions of selected nuclei in the epidermis; yellow arrows indicate nuclei in the
694 mesophyll. Scale bar = 50 μ m.

695



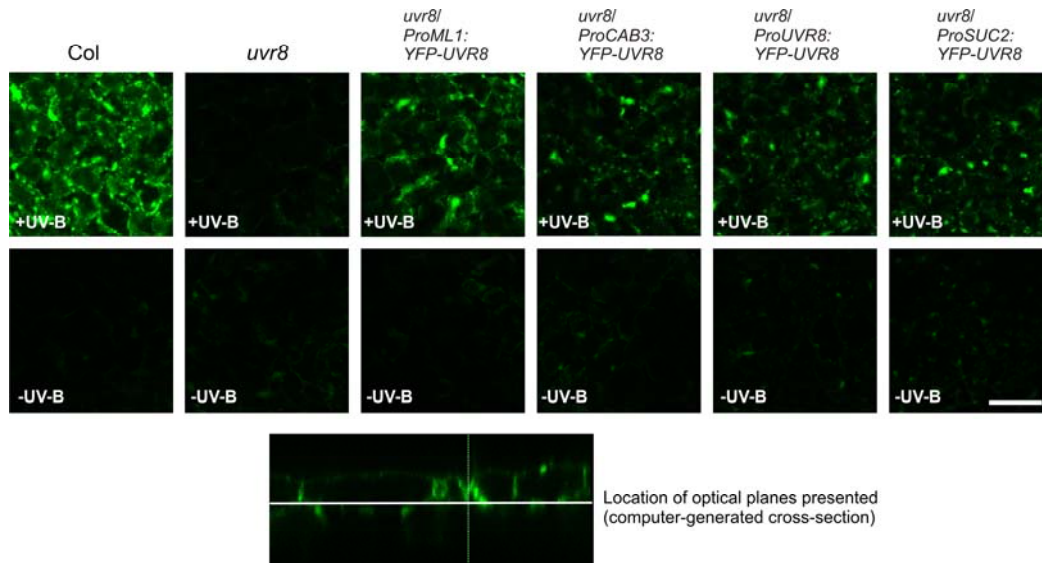
696

697 **Figure 4**

698 **UV-B induction of *ProELIP2:GUS-GFP-NLS* in the cotyledon cells of transgenic**
699 **lines expressing YFP-UVR8 in different tissues.**

700 *ProELIP2:GUS-GFP-NLS* was introduced into transgenic *uvr8-6* lines expressing
701 *ProUVR8:YFP-UVR8* (UVR8), *ProML1:YFP-UVR8* (ML1) or *ProCAB3:YFP-UVR8*
702 (CAB3). Localization of the GUS-GFP-NLS fusion protein was monitored by CLSM
703 in the epidermis and mesophyll cells of the cotyledon of 7-day-old seedlings
704 irradiated with constant WL supplemented with UV-B (+UV-B) or not supplemented
705 (-UV-B). Identical microscope settings were used to allow determination of the
706 difference between the visual signals of the +UV-B and -UV-B image pairs. White
707 arrows mark the positions of selected nuclei in the epidermis, yellow arrows indicate
708 nuclei in the mesophyll. Scale bar = 50 μ m.

709



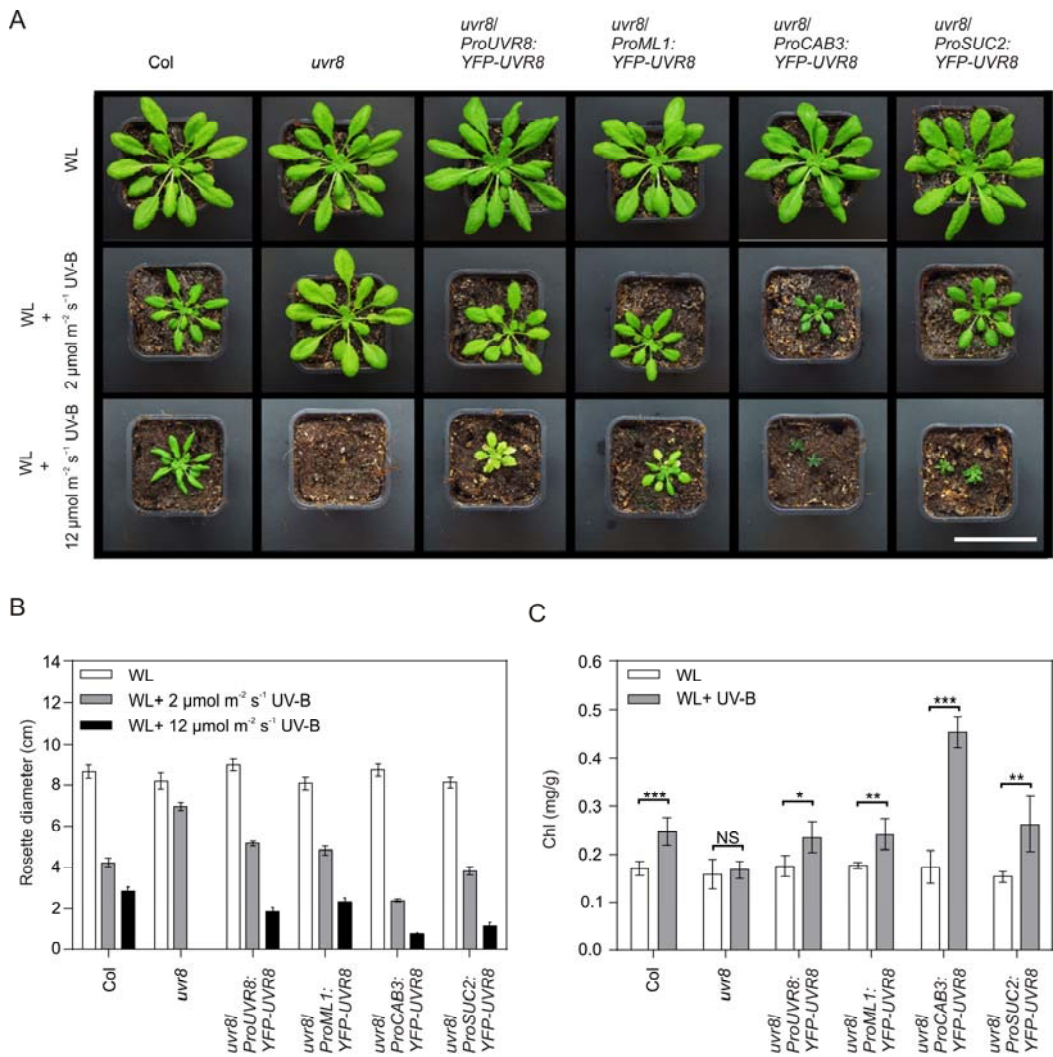
710

711 **Figure 5**

712 **UV-B-induced flavonoid accumulation in the epidermis is regulated by UVR8**
713 **localised in both the epidermis and mesophyll cells.**

714 3-day-old seedlings were grown under WL supplemented with weak UV-B for 4 days
715 and were covered with WG305 (+UV-B) or with WG385 (-UV-B) filter. After

incubation with DPBA, flavonoids were visualized (green colour) using CLSM. All images were taken using the same microscope settings. The focal plane was set to the bottom zone of the adaxial epidermis, where the highest signal was obtained (see bottom panel). Scale bar = 50 μm .



721

722 **Figure 6**

723 **Effect of YFP-UVR8 expressed in different tissues of adult Arabidopsis plants**

724 **A,B. Phenotypic characterization of adult plants grown under white light**

725 **supplemented with UV-B.**

726 A. Phenotypic characterization of 7-week-old *Arabidopsis* plants grown under white
 727 light (WL, $120 \mu\text{mol m}^{-2} \text{s}^{-1}$), WL plus UV-B at $2 \mu\text{mol m}^{-2} \text{s}^{-1}$ or WL plus UV-B at
 728 $12 \mu\text{mol m}^{-2} \text{s}^{-1}$ in short-day conditions. Scale bar: 5 cm.

729 B. Rosette diameter of 7-week-old plants grown as described above. Bars represent
 730 the average values calculated from three independent experiments. Error bars indicate
 731 the standard error of the mean.

732 **C. Chlorophyll content of UV-B irradiated adult plants.**

733 Chlorophyll levels were determined from 7-week-old plants grown under white light
 734 or white light supplemented with UV-B ($1.5 \mu\text{mol m}^{-2} \text{s}^{-1}$) under short day
 735 conditions. Chl (mg/g) represents total chlorophyll content (mg/g fresh weight). Five
 736 plants were used as biological replicates for each line and light treatment. Error bars
 737 indicate standard error of the mean. Asterisks indicate values that are significantly
 738 different from WL treatment in the same genotype (Student's t-test, * $P < 0.05$, ** $P <$
 739 0.01 , *** $P < 0.005$). NS, no significance.

740

741

742 **SUPPORTING INFORMATION**

743 Additional Supporting Information can be found in the online version of this article at
 744 the publisher's web-site:

745 **Table S1**

746 **Complementation of *uvr8* phenotype by different transgenes.**

747 **Figure S1**

748 **Full spectra of the applied light in the GroBank growth chambers**

749 **Figure S2**

750 **Detection of YFP-UVR8 in the cotyledon**

751 **Figure S3**

752 **Detection of YFP-UVR8 in the upper part of the hypocotyl**
753 **Figure S4**

754 **Detection of YFP-UVR8 in the lower part of the hypocotyl.**
755 **Figure S5**

756 **Determination of YFP-UVR8 accumulation in certain tissues.**
757 **Figure S6**

758 **UV-B induction of *ProHY5:GUS-GFP-NLS* in the cotyledon cells of transgenic**
759 **lines expressing YFP-UVR8 in different tissues.**
760 **Figure S7**

761 **The UV-B-specific messenger accumulation of *ELIP2* does whereas the**
762 **accumulation of *PRR9* does not depend on *HY5*.**
763 **Figure S8**

764 **UVB induction of *ProPRR9:GUS-GFP-NLS* in the cotyledon cells of transgenic**
765 **lines expressing YFP-UVR8 in different tissues.**
766 **Figure S9**

767 **Determination of endogenous and YFP-UVR8 protein levels in adult plants.**

768 **SUPPORTING INFORMATION**

769

770 Table S1

771 Figures S1-S9

772

773 **Expression of UVR8 photoreceptor in different tissues reveals tissue-autonomous**

774 **features of UV-B signalling**

775

776 Péter Bernula, Carlos Daniel Crocco, Adriana Beatriz Arongaus, Roman Ulm, Ferenc

777 Nagy, András Viczián

778

line and transgene activity	seedling phenotype			adult phenotype	
	hypocotyl	cotyledon	flavonoid accumulation	rosette size	chlorophyll content
Col	+++	+++	+++	+++	+++
<i>uvr8</i>	-	-	-	-	-
<i>uvr8/ProUVR8:YFP-UVR8</i> (epidermis, subepidermis)	++	+++	++	++	++
<i>uvr8/ProML1:YFP-UVR8</i> (epidermis)	++	+++	++	++	++
<i>uvr8/ProCAB3:YFP-UVR8</i> (subepidermis)	++	-	++	+++	+++
<i>uvr8/ProSUC2:YFP-UVR8</i> (subepidermis, vasculature)	+	-	++	+++	+++

780

781 **Table S1**782 **Complementation of *uvr8* phenotype by different transgenes.**

783 This table summarizes the results obtained from different phenotype analyses. Crosses
784 mark the level of *uvr8* mutant complementation. Wild type (Col) plants show fully
785 extended responses (+++) whereas *uvr8* mutant shows no UVR8-specific responses in
786 the assays (-). The first column also indicates the tissue types where the YFP-UVR8
787 was observed by CLSM.

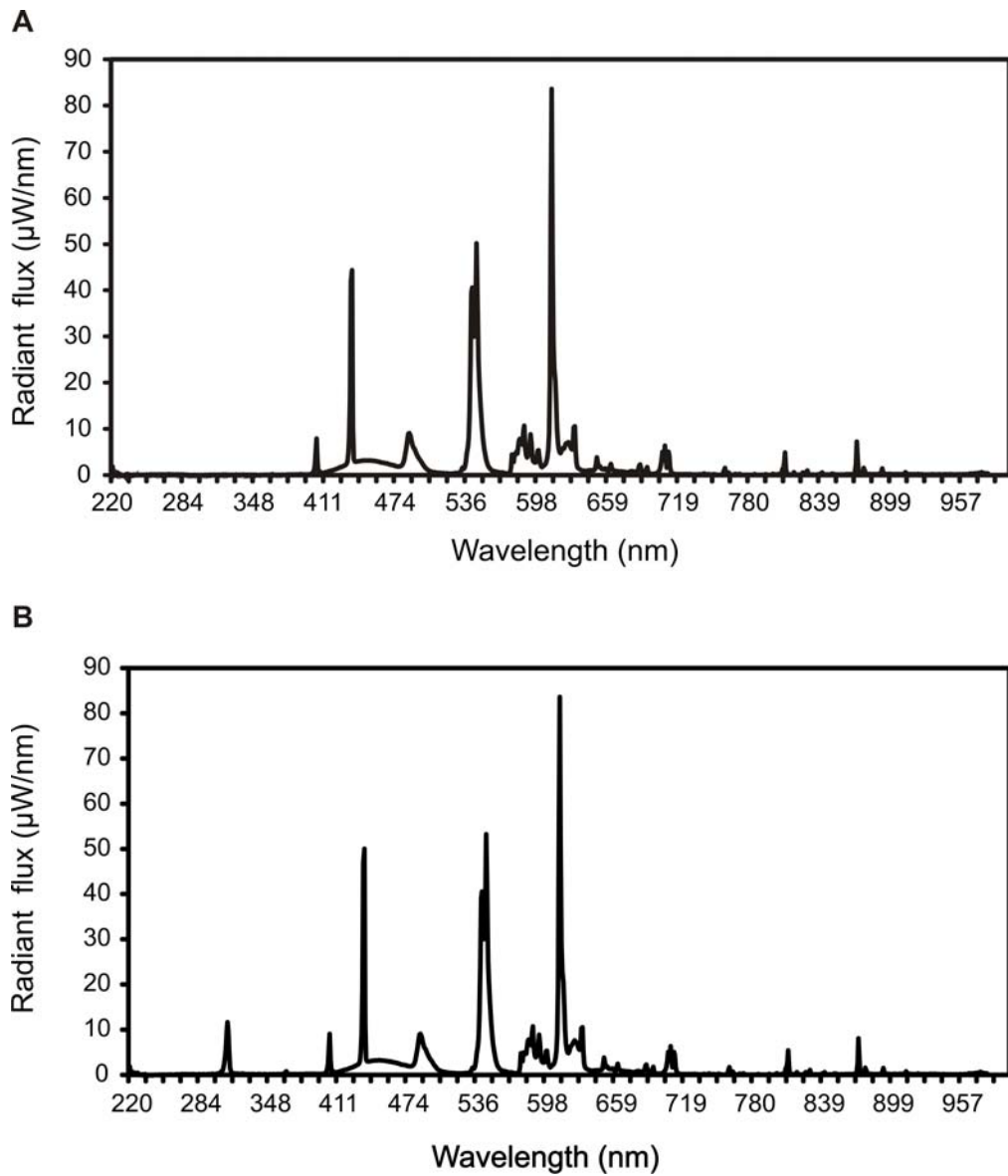


Figure S1

Full spectra of the applied light in the GroBank growth chambers

A. White light only.

B. White light supplemented with UV-B.

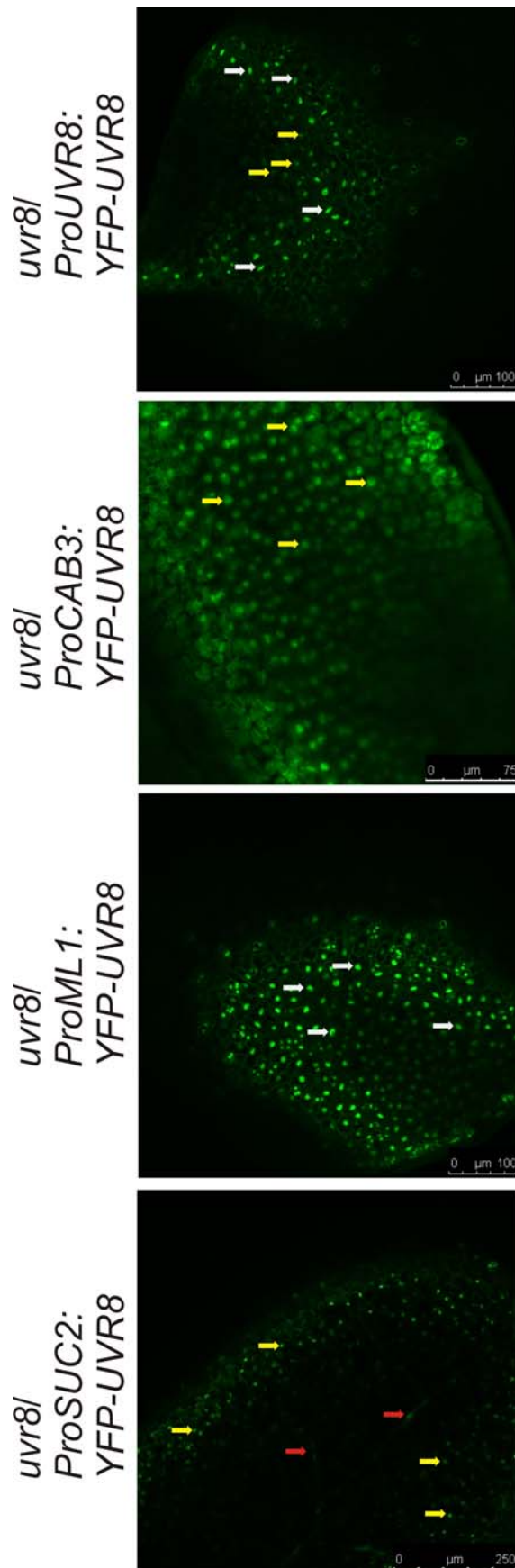


Figure S2

Detection of YFP-UVR8 in the cotyledon.

Localization of YFP-UVR8 fusion protein in *uvr8-6* mutant seedlings expressing *ProUVR8:YFP-UVR8*, or *ProML1:YFP-UVR8* or *ProCAB3:YFP-UVR8* or *ProSUC2:YFP-UVR8* was monitored by confocal laser scanning microscopy in the cotyledons of seedlings grown for 4 days in constant white light supplemented with UV-B. Images were obtained using identical microscope settings and YFP-specific signal is presented. White arrows mark positions of selected nuclei in the epidermis, yellow arrows point at nuclei in the mesophyll whereas red arrows indicate nuclei/cells in the vasculature.

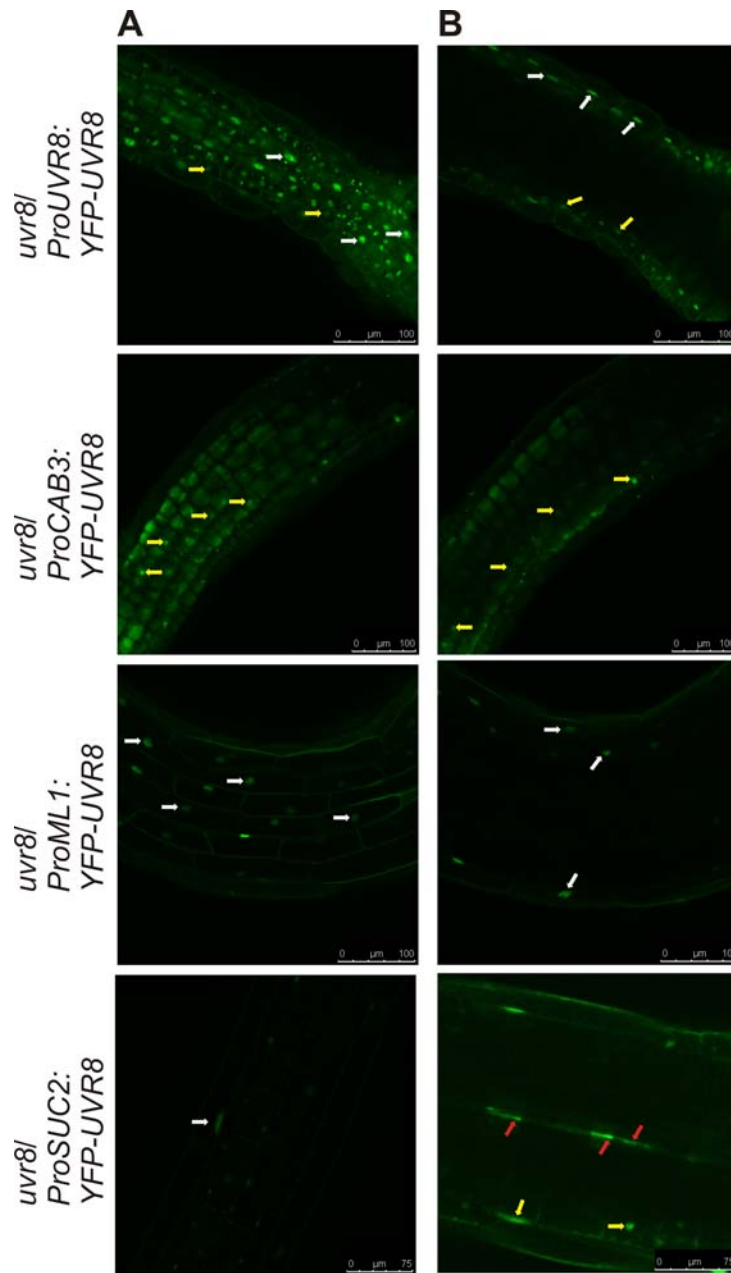


Figure S3

Detection of YFP-UVR8 in the upper part of the hypocotyl.

A. Focus was set to the epidermis and subepidermis.

B. Focus was set to the subepidermis and vasculature.

Localization of the YFP-UVR8 fusion protein was monitored by CLSM in the upper part of the hypocotyls of seedlings grown for 4 days in constant white light supplemented with UV-B. Images were obtained using identical microscope settings, with the exception of images taken of the *ProSUC2:YFP-UVR8* line which is presented with enhanced signal for better visibility. White arrows mark positions of selected nuclei in the epidermis, yellow arrows point at nuclei in the mesophyll whereas red arrows indicate nuclei/cells in the vasculature.

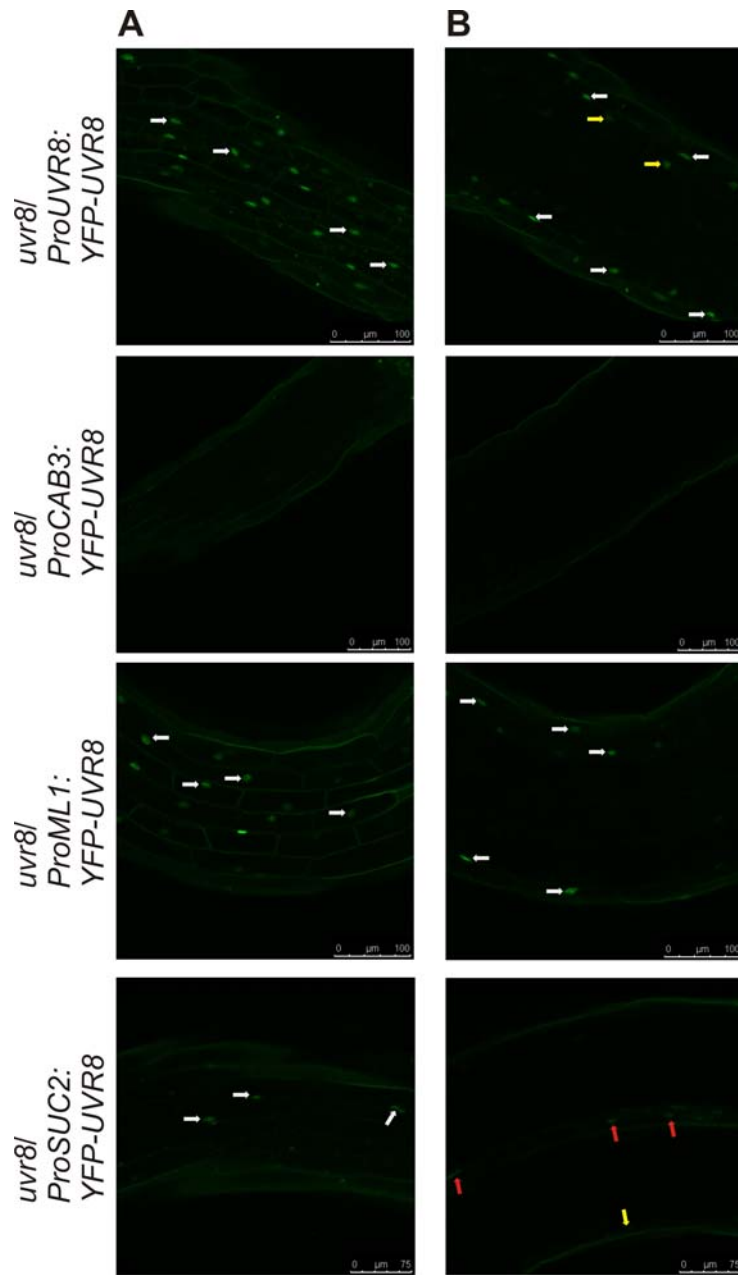


Figure S4

Detection of YFP-UVR8 in the lower part of the hypocotyl.

A. Focus was set to the epidermis and subepidermis.

B. Focus was set to the subepidermis and vasculature.

Localization of the YFP-UVR8 fusion protein was monitored by CLSM in the lower part of the hypocotyls grown for 4 days in constant white light

supplemented with UV-B.

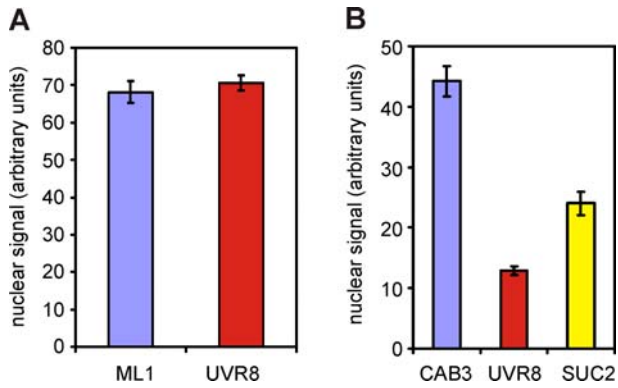
Images were obtained using identical microscope settings.

White arrows mark positions of selected nuclei in the epidermis;

yellow arrows point at nuclei in the mesophyll whereas red

arrows indicate nuclei/cells in the vasculature.

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Figure S5

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Determination of YFP-UVR8 accumulation in certain tissues.

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Nuclear YFP-UVR8 signal was determined using confocal laser scanning microscopy.

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uvr8-6 seedlings expressing (A) *ProUVR8:YFP-UVR8* (UVR8) and *ProML1:YFP-*

807

UVR8 (ML1) transgenes in the epidermal cell layer of the cotyledon (B)

808

ProUVR8:YFP-UVR8 (UVR8) and *ProCAB3:YFP-UVR8* (CAB3) *ProSUC2:YFP-*

809

UVR8 (SUC2) transgenes in the subepidermal cell layer of cotyledon were examined

810

using identical microscope settings. Error bars indicate standard error of the mean

811

(n>60).

812

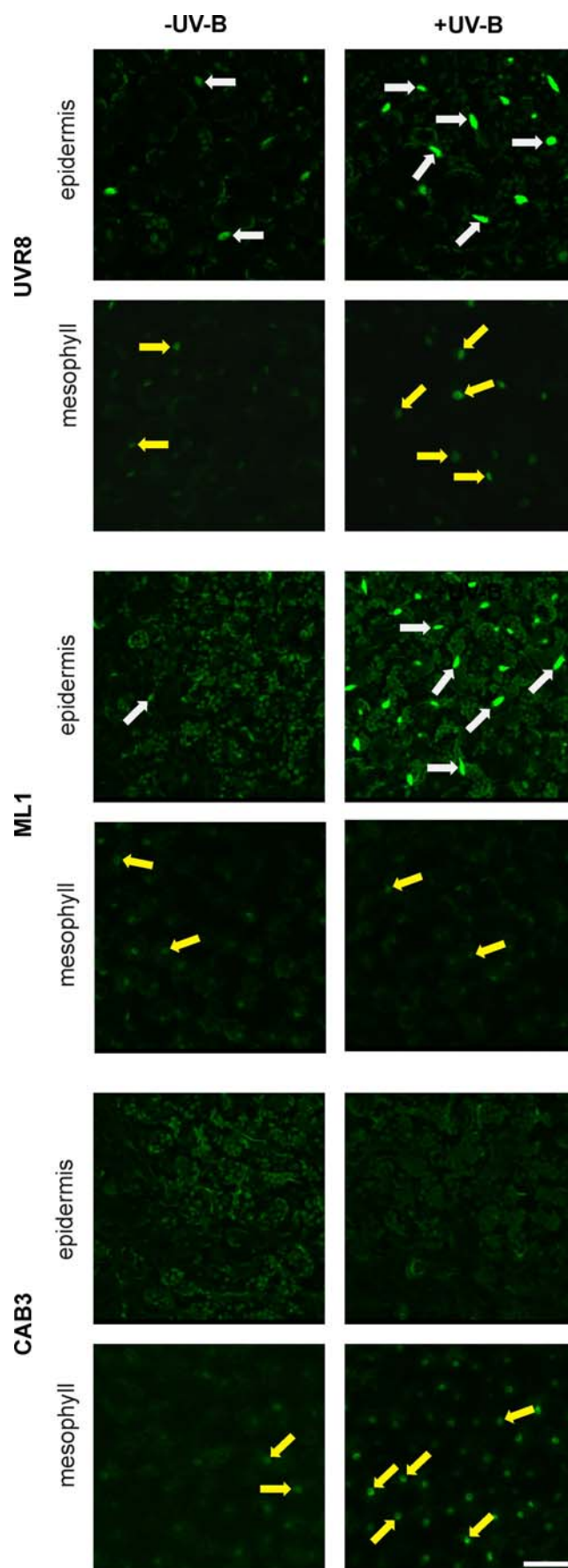
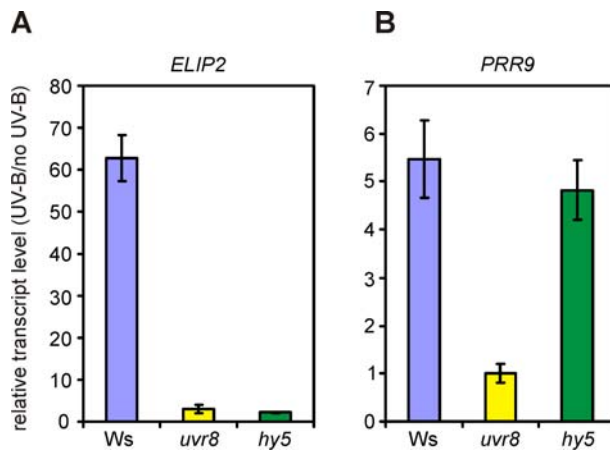


Figure S6

UV-B induction of *ProHY5:GUS-GFP-NLS* in the cotyledon cells of transgenic lines expressing YFP-UVR8 in different tissues.

ProHY5:GUS-GFP-NLS was introduced into transgenic *uvr8-6* lines expressing *ProUVR8:YFP-UVR8* (UVR8) *ProML1:YFP-UVR8* (ML1) or *ProCAB3:YFP-UVR8* (CAB3). Localization of the GUS-GFP-NLS fusion protein was monitored by confocal laser scanning microscopy in the epidermis and mesophyll cells of the cotyledon of 7-day-old seedlings irradiated with constant WL supplemented with (+UV-B) or without (-UV-B) UV-B ($1.5 \mu\text{mol m}^{-2} \text{sec}^{-1}$). Identical microscope settings were used to allow the visualisation signal difference between the +UV-B and -UV-B image pairs. White arrows mark positions of selected nuclei in the epidermis; yellow arrows indicate nuclei in the mesophyll. Scale bar = 50 μm .

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Figure S7

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The UV-B-specific messenger accumulation of *ELIP2* does whereas the accumulation of *PRR9* does not depend on *HY5*.

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7-day-old seedlings were irradiated with a 90 min WL pulse mixed with UV-B. Half

822

of the seedlings were covered with WG305 filter (UV-B), the other half was covered

823

with WG385 filter (no UV-B). After the irradiation, seedlings were collected and

824

transcript level of *ELIP2* (A) and *PRR9* (B) was determined using quantitative RT-

825

PCR. The relative transcript level (UV-B/no UV-B) is plotted in the figure. Ws=

826

Wassilewskaya wild type; *uvr8*= *uvr8-7* mutant; *hy5*= *hy5-ks50* mutant (both in Ws

827

ecotype).

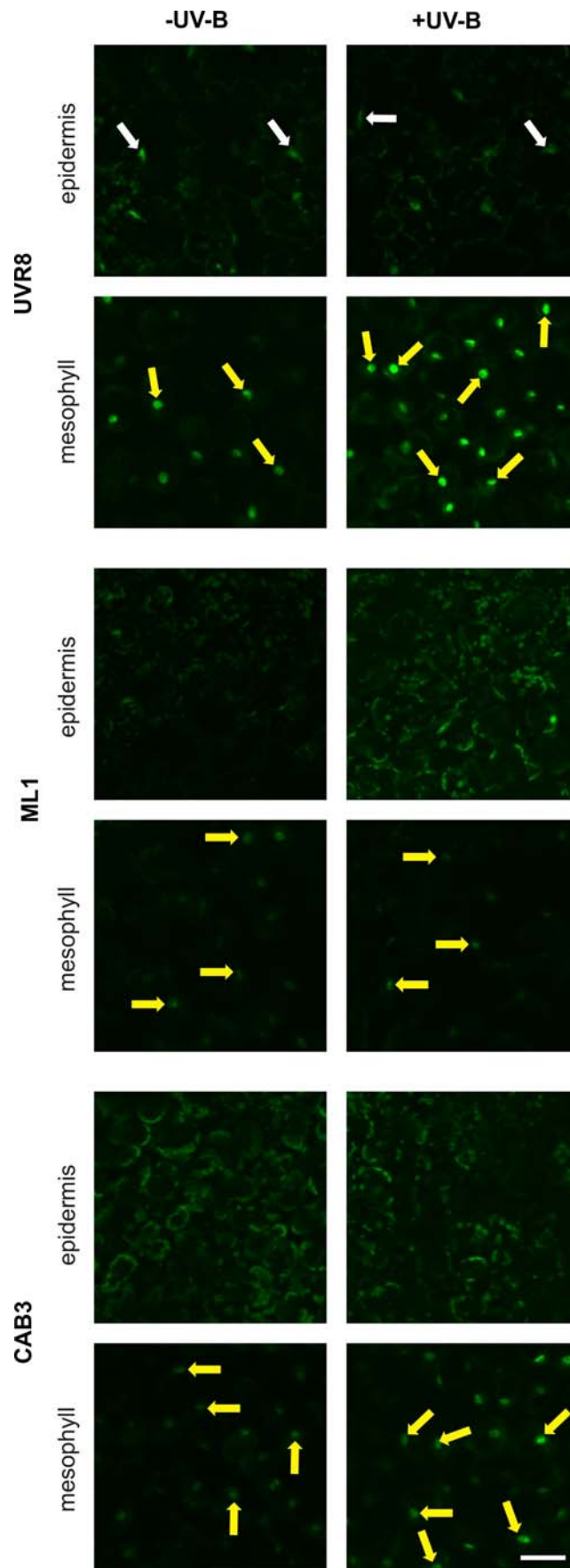


Figure S8

UVB induction of *ProPRR9:GUS-GFP-NLS* in the cotyledon cells of transgenic lines expressing YFP-UVR8 in different tissues.

ProPRR9:GUS-GFP-NLS was introduced into transgenic *uvr8-6* lines expressing *ProUVR8:YFP-UVR8* (UVR8) *ProML1:YFP-UVR8* (ML1) or *ProCAB3:YFP-UVR8* (CAB3). Localization of the GUS-GFP-NLS fusion protein was monitored by CLSM in the epidermis and mesophyll cells of the cotyledon of 7-day-old seedlings irradiated with constant WL supplemented with (+UV-B) or without UV-B (-UV-B). Identical microscope settings were used to allow the visualisation signal difference between the +UV-B and -UV-B image pairs. White arrows mark positions of selected nuclei in the epidermis, yellow arrows indicate nuclei in the mesophyll. Scale bar = 75 μ m.

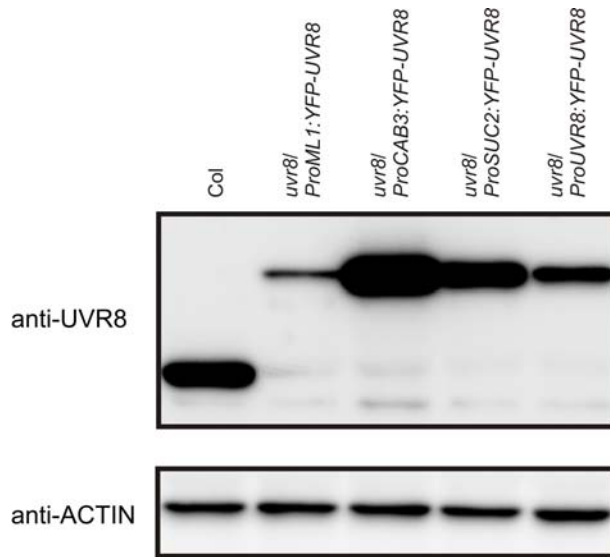


Figure S9

Determination of endogenous and YFP-UVR8 protein levels in adult plants.

Total protein extract was isolated from 7-week-old seedlings grown under short day conditions in the greenhouse. Proteins were separated by gel electrophoresis blotted onto a membrane and hybridized with anti-UVR8-specific antibody (anti-UVR8). The blot was reprobed with anti-ACTIN antibody to check the even loading.